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Stimulation of Inositol 1,4,5-Tris Phosphate and Endothelin-1 Receptor Binding in Rats Treated with Cyclosporin-A[1]

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Cyclosporin-A (CsA) is known to induce alterations in cellular Ca^{2+} homeostatsis by increasing transmembrane Ca^{2+}-influx and intracellular mobilization from Ca^{2+} Stores. We studied the *in vitro* and *in vivo* effects of CsA on intracellular second messengers like inositol 1,4,5-trisphosphate ($InsP_3$) and endothelin-1 (ET-1) receptor binding in rat brain and kidney particulate fractions. CsA in concentrations of 10-1000 μg stimulated rat brain and kidney IP_3 and ET-1 receptor binding *in vitro* in a concentration dependent manner and changes were statistically significant for most of the higher concentrations of the drug. Rats treated with 10 or 25 mg/kg body weight of CsA also showed significant elevation in their IP_3 and ET-1 receptor binding. These results suggest that CsA may induce its toxic effects by interfering with second messenger pathways that are involved in mobilization of intracellular Ca^{2+}.

Key Words : Cyclosporin A, Inositol 1,4,5-trisphosphate ($InsP_3$), Endothelin-1 (ET-1), Rat

Introduction

Cyclosporin-A (CsA 1202, Log P + 2.99) is a cyclic immunosuppressant peptide of fungal origin which acts by blocking the sensitization of T-lymphocytes in the immune process (Borel et al. 1977). It is accumulated by most body cells and at high intracellular concentration it is cytotoxic (Begley 1992). Nephrotoxicity and arterial hypertension are the main side effects of CsA (Meyer & Schrier 1988, Schachter 1988, Koop & Klotman 1990). Numerous toxicologic studies have indicated that over dosage of CsA leads to hepatotoxicity and nephrotoxicity (Ryffel et al. 1983) and upon CsA toxicity, lipid peroxidation, high energy metabolism

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and cytosolic toxicity/Ca^{2+} are being affected (Triffilis & Kahug 1988). Alterations in Ca^{2+} homeostasis upon CsA induced toxicity has been well established (Triffilis & Kahug 1988, Baldari et al. 1991, Altschuld et al. 1992) and especially CsA's interference of receptor operated Ca^{2+} channels has also been reported (Mason 1989, Mc Nally et al. 1991, Awazu et al. 1991, Takeda et al. 1992). In view of the key role played by inositol 1,4,5 tris-phosphate (IP_3) and endothelin-1 (ET-1) receptors in regulation of Ca^{2+} mediated events, we present here the data concerning to *in vitro* and *in vivo* effects of CsA on rat brain and kidney IP_3 and ET-1 receptor binding.

Materials and Methods

Cyclosporin-A was purchased from Sigma Chemical Co., St., Louis, Missouri (^3H)-inositol 1,4,5 trisphosphate (I^{125})-endothelin-1 were purchased from New England Nuclear corporation, Boston, MA. All other chemicals were of technical grade obtained from Sigma Co. Sprague-Dawley adults rats (200-250 g) were obtained from Jackson State Laboratories (USA) and housed in cages for one week before experimental treatment was initiated.

Treatment of Animals

Stock solutions of CsA were prepared in 96% ethanol (500 mg of CSA/400 µl of ethanol and required dilutions (10-1000 µg) were made in saline. Animals received 10 or 25 mg/kg body weight of CsA/animal/ 4 weeks (Weekly IP injections), control animals (n=4) received equal amounts of ethanol + saline (0.5% ethanol in 0.9% saline. For *in vitro* studies CsA in conc. of 10-1000 µg was used. Ethanol in concentrations as low as 0.5 per cent used to incubate/treat the experimental samples has no effect on either IP_3 or ET-1 receptor binding.

Animals were sacrificed by decapitation and brain and kidney were quickly isolated, frozen in liqid nitrogen and stored at –80°C until used.

(^3H)-IP3 Receptor Binding Assay

The method of Worley et al. (1987) was used for IP_3 receptor studies using (^3H)-IP_3. Briefly tissues were homogenized in 10 volumes of tris buffer (50 mM Tris HCl, 1 mM EDTA, *p*H 8.3) and centrifuged at 700 g for 5 min. The supernatant was centrifuged at 35000 g for 10 min. and the pellet was suspended in the above buffer and this membrane preparation was used in IP_3 binding studies. The reaction mixture contained 600 mg protein, 2.5 nm (^3H)-IP_3 (lci/m mole) in a total volume of 1 ml. The incubation was for 10 min at 4°C and the reaction was stopped by centrifugation at 10000 g for 10 min followed by aspiration of the supernatant. The non-specific binding was determined as described for specific receptor binding except the presence of unlabelled IP_3. The radioactivity in the pellets was determined by suspending the pellet in 10 ml of aquasol and counting in a liquid scintillation counter (Beckman LS 6000 TA).

ET-1 Receptor Binding

ET-1 receptor binding was determined by Bolger et al. (1990). The tissue was homogenized in 5 volumes of Tris-PSS buffer (25 mM *Tris* HCl, 135 mM NaCl, 2.7 mM KCl, 1.8 mM $CaCl_2$, 1.1 mM $MgSO_4$, *p*H 7.4). The homogenates were centrifuged at 1000 g for 10 min and the supernatant was recentrifuged at 22000 g for 10 min. The pellet was suspended in Tris-PSS buffer and this membrane preparation was used for the

ET-1 receptor binding studies. ^{125}I binding was initiated in polypropylene tubes in a total volume of 1 ml consisting of radioligand, membrane fraction, 5 μM aprotinin, 1 μM phenyl methyl sulfonyl fluoride (PMSF) and Tris PSS. Tubes were incubated for 50 min at 20°C and the binding was terminated by centrifugation at 10000 g for 10 min followed by aspiration of the supernatant. The pellets were transferred to scintillation vials containing 2 ml aquasol and their radioactivity determined. The non-specific binding was determined by adding unlabelled ET (10^{-7} M) in the binding assay before addition of (^{125}I) ET-1.

Protein content of the samples was measured by Bio-rad protein assay kit and gamma gloublin was used as standard.

Statistical Analysis

Statistical analysis was done using a computer programme that calculates significance using two samples t-test. A value of $P < 0.005$ was accepted as statistically significant. Each assay was performed in duplicates and a minimum of 3-6 animals were employed in each treatment. The values were expressed as F mol/mg protein/minute.

Results and Discussion

CsA in the concentration range of 10-1000

Table 1 *Effect of cyclosporin — A on rat brain and kidney ET-1 and IP_3 receptor binding in vitro. (Values expressed as F mol/mg protein/minute)*

		Concentration of Drug (μg)					
Tissue		Control	10	50	100	500	1000
Brain		768.33	801.26	824.27	867.62	933.75	1138.49
	ET-1	±52.31	±46.47	±40.58	±57.98	±51.75	±63.92
			NS	NS	NS	S	S
	IP_3	680.52	692.31	745.31	810.74	1050.30	1277.57
		±70.26	±45.76	±103.89	±74.66	±151.79	±64.48
			NS	NS	NS	S	S
Kidney		158.50	178.65	224.56	290.50	324.34	359.25
	ET-1	±4.136	±23.69	±16.09	±19.91	±42.84	±36.78
			NS	NS	VS	S	S
	IP_3	180.82	183.70	190.16	283.02	423.36	5111.57
		±28.46	±37.89	±21.05	±67.33	±33.04	±21.42
			NS	NS	NS	S	S

Each value is the mean ± SD of 3 experiments assayed in duplicate.
S - Significant
VS - Very significant

μg stimulated ET-1 and IP_3 receptor binding of both rat brain and kidney *in vitro* in a concentration dependent manner of the drug and changes observed were statistically significant ($P<0.05$ (or) $P<0.005$) for 500 and 1000 μg concentrations of CsA (table 1). Rats receiving 10 or 25 mg/kg body-weight of CsA also exhibited stimulation of IP_3 and ET-1 receptor binding *in vivo* and in the 25 mg/kg body weight of CsA treated group the changes were statistically significant ($P<0.005$) over their control values (table 2).

The results of this study has clearly demonstrated that IP_3 and ET-1 receptor binding in rat brain and kidney were stimulated both *in vitro* and *in vivo* by CsA.

Table 2 — *Effect of cyclosporin A on rat brain and kidney ET-1 and IP_3 receptor binding in vivo (Values expressed as F mol/mg protein/ minute).*

Name of Tissue	Control	10 mg/kg	25 mg/kg
Brain ET-1	387.90	331.44	549.47
	±73.41	±62.60	±62.18
		NS	VS
IP_3	192.95	243.60	373.65
	±40.68	±107.56	±112.60
		NS	VS
Kidney ET-1	186.95	149.60	291.38
	±11.92	±9.65	±23.57
		VS	VS
IP_3	167.18	164.5	235.42
	±18.80	±28.50	±38.85
		NS	VS

Each value in the mean ± SD of 6 experiments assayed in duplicate. NS: Not significant, VS-Very significant

This ultimately may result in release of Ca^{2+} from intracellular stores. Similar interaction of metals like lead with in $InsP_3$ of rat brain (Vig et al. 1994) and drugs like anthracyclines with ET-1 and IP_3 receptors in various organs of rats have been reported (Veeri Setty et al. 1994) earlier.

$InsP_3$ binds to a specific $InsP_3$ receptor and induces the release of Ca^{2+} into the cytoplasm from intracellular storage sites (Furuchi et al. 1992). ET's are regulatory peptides that binds to receptors on cell membranes and mediate Ca^{2+} release in cellular systems (Simunson & Dunn 1990). CsA has been shown to interfere with biological membranes (Koppe et al. 1990) and alter Ca^{2+} transporters and also certain properties of cell membranes (O'Leardy et al. 1986). It is reported elsewhere that CsA alter Ca^{2+} mediated events of cellular systems (Mathus et al. 1986), particularly increase in Ca^{2+} flux and intracellular Ca^{2+} in the presence of CsA have been emphasized in number of studies (Pfeilschifter & Ruegg 1987, Meyer-Lehnert & Schrier 1988, Nacchia et al. 1988). Increased intracellular Ca^{2+} may result either by increased influx or decreased efflux of Ca^{2+} across plasma membrane or via increased release of stored Ca^{2+} from mitochondrial or enodplasmic storage sites through involvement of receptor operated channels (Mc Nally et al. 1991). The possibility of ET-1 like receptors to contribute for increase of cytosolic Ca^{2+} upon CsA administration has been well established (Takeda et al. 1992). Owing to interaction of CsA with cell membranes and cellular Ca^{2+} homeostasis, it is likely that it may interfere with ET-1 as well as IP_3 receptor mediated events of rat kidney and brain as supported by our present study. Significant changes observed in rat kidney and brain

ET-1 and IP_3 receptors in our present study reveal that these membrane bound receptors were more sensitive to a CsA concentration of 25 mg/kg body wt, and that of kidney ET-1 receptors in particular appears to be sensitive to a very low concentration of CsA like 10 mg/kg body wt. Since the presence of ET-1 and IP_3 receptor sites in various organs of mammals is well known (Simonson et al. 1990 and Furuchi et al. 1992) stimulation of both ET-1 and IP_3 receptor binding in rat brain and kidney upon CsA administration might contribute for release of Ca^{2+} from intracellular stores and this inturn may result in raise of intracellular Ca^{2+} in the presence of CsA (Mc Nally et al. 1991) and all these changes in part may contribute for CSA induced nephrotoxicity and neurotoxicity.

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